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A Putative Gene Cluster for Aminoarabinose Biosynthesis Is Essential for *Burkholderia cenocepacia* Viability


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Cationic antimicrobial peptides (APs), a group of structurally diverse molecules found in all eukaryotes (17, 34), can kill gram-positive and gram-negative bacteria, fungi, and viruses and also modulate innate immune responses (30). In gram-negative bacteria, APs interact with lipopolysaccharide (LPS) and via the self-promoted uptake pathway reach targets in the plasma membrane (16). LPS is a major surface component of gram-negative bacteria, consisting of O antigen polysaccharide, core oligosaccharide, and lipid A (29). Phosphate groups covalently attached to residues on the lipid A and core oligosaccharide provide sites for electrostatic interactions with APs (18). Bacteria can resist APs by modifying their lipid A with 4-amino-4-deoxy-l-arabinose (Ara4N), effectively reducing the net negative charge of LPS molecules. In all species examined to date, this is a highly regulated process in response to environmental Mg²⁺ limitation, excess Fe⁴⁺, or the presence of APs (1, 8, 9, 12, 15, 24). However, LPS modification with Ara4N is dispensable for growth in vitro under routine laboratory conditions.

*Burkholderia cenocepacia* belongs to the *B. cepacia* complex, a group of closely related environmental bacteria that are emerging opportunistic pathogens of cystic fibrosis patients and other immunocompromised individuals (23). They are highly resistant to the majority of clinically useful antimicrobials and also resistant to APs even at concentrations that kill other bacteria with Ara4N-modified LPS (21). Very little is known about the mechanism of resistance to APs in *Burkholderia* spp. LPS from various *Burkholderia* species, such as *B. cepacia*, *B. caryophylli*, and *B. cenocepacia*, contains Ara4N residues in the lipid A and also the unusual *glycero-talo*-octulosonic acid (Ko) residue in the inner-core oligosaccharide (10, 13, 19, 20, 25, 26, 31, 32). Here, we show that a putative gene cluster for Ara4N biosynthesis and LPS modification is essential for the viability of *B. cenocepacia*.

Using a conditional mutagenesis strategy we demonstrate here that a gene cluster encoding putative aminoarabinose (Ara4N) biosynthesis enzymes is essential for the viability of *Burkholderia cenocepacia*. Loss of viability is associated with dramatic changes in bacterial cell morphology and ultrastructure, increased permeability to propidium iodide, and sensitivity to sodium dodecyl sulfate, suggesting a general cell envelope defect caused by the lack of Ara4N.

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The putative Ara4N biosynthesis cluster is essential for viability of *B. cenocepacia*. Repeated attempts to mutagenize *arnT*, *arnB*, *arnC*, and *BCAL1935* by insertional inactivation using the pGP1TPp suicide plasmid (11) containing internal fragments from each target gene consistently failed. In contrast, the genes *BCAL1928* and *BCAL1936* that flank each end of the putative *arn* cluster were readily mutated. We hypothesized that the putative *arn* genes perform one or more functions that are essential for the viability of *B. cenocepacia*. To confirm the essentiality of the *arn* genes, we constructed plasmid pSC200, which enables the delivery of the rhamnose-inducible *PrhaB* promoter into the chromosome to drive the expression of a targeted gene. The construction of pSC200 was done by combining the multiple-cloning site, ori*R6K*, and *mob* genes from pGp1TPp (11) with the *PrhaB* rhamnose-inducible promoter, *rhaR*, *rhaS*, and the *dhfr* cassette from pSChraB2 (7). Details on the construction of this plasmid are available from the authors upon request. Several 300-bp fragments spanning the 5' region of each targeted gene were cloned into pSC200. The resulting plasmids were transferred into *B. cenocepacia* K56-2 by triparental mating, and the exconjugants were subjected to the same transcriptional regulation as has been observed in all other bacteria examined to date (1, 24). To facilitate genetic manipulations the remaining studies were performed using strain K56-2, which is also highly resistant to APs (21).

FIG. 1. (A) Genetic organization of the putative *arn* gene cluster and flanking regions on chromosome 1 of *B. cenocepacia* strains J2315 and K56-2. Solid boxes around gene and locus names indicate the genes for which polar mutations failed; dotted boxes indicate genes for which the creation of polar mutations was successful. Mutagenesis of the remaining genes was not attempted. The dotted arrows indicate the locations of the inserted rhamnose-inducible promoter in strains XOA10, XOA11, and XOA12. Black and gray arrows denote the genes within the two transcriptional units of the putative *arn* cluster that were identified by RT-PCR. (B) RT-PCR analysis of the *arn* cluster, performed on strain J2315 in the absence of polymyxin B challenge.

RT-PCR analysis also revealed that the *arn* cluster in both strains was expressed regardless of polymyxin B challenge (data not shown), indicating that it is not subjected to the same transcriptional regulation as has been observed in all other bacteria examined to date (1, 24). To confirm the essentiality of the putative *arn* cluster that were identified by RT-PCR, (A) Genetic organization of the putative *arn* gene cluster and flanking regions on chromosome 1 of *B. cenocepacia* strains J2315 and K56-2. Solid boxes around gene and locus names indicate the genes for which polar mutations failed; dotted boxes indicate genes for which the creation of polar mutations was successful. Mutagenesis of the remaining genes was not attempted. The dotted arrows indicate the locations of the inserted rhamnose-inducible promoter in strains XOA10, XOA11, and XOA12. Black and gray arrows denote the genes within the two transcriptional units of the putative *arn* cluster that were identified by RT-PCR. (B) RT-PCR analysis of the *arn* cluster, performed on strain J2315 in the absence of polymyxin B challenge.
therefore concluded that expression of one or more genes in the two transcriptional units of the putative arn cluster is indispensable in *B. cenocepacia* K56-2.

The first biochemical reaction for the biosynthesis of Ara4N is the conversion of UDP-glucose into UDP-glucuronic acid by the enzyme UDP-glucose 6-dehydrogenase (Ugd or PmrE). In previous work, we characterized a gene operon containing a *ugd* homologue (BCAL2946) and genes encoding modification of *glycero-manno*-heptose, which are part of the biosynthesis of the ADP-*glycero-manno*-heptose precursor for core oligosaccharide biosynthesis (21). To assess whether or not *ugd*BCAL2946 is also essential, we constructed a conditional *ugd*BCAL2946 mutant (SAL10). This mutant did not show a conditional lethal phenotype in medium with glucose (Fig. 2A), indicating that *ugd*BCAL2946 is not essential for bacterial viability. A BLAST analysis of the *B. cenocepacia* J2315 genome revealed the presence of two additional *ugd* homologues in chromosome 2 (BCAM0855 and BCAM2034), which we assume provide redundant UDP-glucose dehydrogenase activities.

Loss of viability is associated with morphological changes in the bacterial cells and defects in cell envelope permeability. The essentiality of Ara4N synthesis was further demonstrated by depletion experiments performed using liquid medium (Fig. 2B). Eight-hour cultures were diluted to an OD600 of 0.001 in M9 medium supplemented with 5% (wt/vol) yeast extract and 0.5% (wt/vol) rhamnose and incubated at 37°C for 11 h. Cells from these cultures were spun down, washed three times with sterile PBS, and diluted with fresh M9 medium supplemented with 5% (wt/vol) yeast extract and 0.5% (wt/vol) glucose at a final OD600 of 0.13. Growing cells were subcultured with fresh medium containing 0.5% (wt/vol) glucose at 4 h and monitored for growth for another 6 h. Growth curves of the wild-type strain and all the mutants in permissive conditions were identical (data not shown). After serial passages in medium with glucose, the wild type and the XOAI0 mutant also grew to similar levels (Fig. 2B). In contrast, after serial passage in medium with glucose of XOAI1 and XOAI2 cultures, the turbidity of the cultures rapidly reached a plateau compared to wild-type and XOAI0 control strain results. The results indicate that XOAI1 and XOAI2 had stopped growing after 6 h (roughly corresponding to six generations). Phase-contrast microscopy and fluorescent staining with Syto9 and propidium iodide (LIVE/DEAD BacLight bacterial viability kit; Molecular Probes, Invitrogen Detection Technologies, Eugene, OR) were carried out to investigate the bacterial cell morphology and to assess whether or not the cell envelope was compromised. After 8 h in nonpermissive conditions, strains XOAI1 and XOAI2, but not K56-2 and XOAI0, formed chains of cells and the cultures contained flocculent particulate material indicative of cell lysis. Also, 50% of XOAI1 and 58% of XOAI2 bacterial cells were permeable to propidium iodide in contrast to 5% to 10% of the control strains, suggesting a loss of viability and a compromised cell envelope in the *arn* conditional mutants (Fig. 3). No differences in morphology or viability were observed between these same strains grown overnight under permissive conditions before being transferred to the nonpermissive medium for the depletion experiments (data not shown).

Ultrastructural analysis of bacterial cells after 8 h following depletion revealed accumulations of membranous material in strains XOAI1 and XOAI2 (Fig. 4), suggesting that Ara4N is required for the proper assembly of the outer membrane. Furthermore, empty cells and cells with cell division defects could be observed in the mutant cultures (Fig. 4). To obtain further evidence that the envelope was compromised in XOAI1 and XOAI2 under nonpermissive conditions, bacteria were grown in the presence of 0.05% (wt/vol) sodium dodecyl sulfate (SDS). For these experiments, cells were diluted to an OD600 of 0.1 and dilutions were plated for colony counts on M9 agar plates supplemented with 5% (wt/vol) yeast extract, 0.5% (wt/vol) rhamnose, and Tp (100 µg/ml) with or without 0.05% (wt/vol) SDS. The survival rate of K56-2 cells in the presence of SDS was 1.2% of the initial inoculum, while the survival rates of XOAI1 and XOAI2 cells were 0.09% and 0.08%, respectively. Together, these experiments demonstrate that the putative *arn* gene cluster is essential for *B. cenocepacia* viability and that its function is required for an intact cell envelope. It is possible that LPS modifications with Ara4N are an essential requirement for the stability of the outer membrane in this
FIG. 3. Microscopy and live-dead staining of strains K56-2, XOA10, XOA11, and XOA12 at 8 h of growth during the depletion experiments. Live bacteria appear fluorescent green, while dead bacteria and bacteria with compromised membranes appear fluorescent red. These experiments were carried out twice with similar results.
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